



Aberrant tryptophan transport in cultured fibroblast from patients with Male Idiopathic Osteoporosis: An in vitro study

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ABSTRACT

It has been demonstrated, that long-term chronic tryptophan deficiency, results in decreased serotonin synthesis, which may lead to low bone mass and low bone formation. Findings from studies in male patients with idiopathic osteoporosis suggested a decreased transport of tryptophan in erythrocytes of osteoporotic patients, indicating that serotonin system defects may be involved in the etiology of low bone mass. Tryptophan is the precursor of serotonin, and a disturbed transport of tryptophan is implicated in altered serotonin synthesis. However, no study has investigated the tryptophan transport kinetics in MIO patients. The aim of this study is to investigate the kinetic parameters of tryptophan transport in fibroblasts derived from MIO patients compared to age and sex matched controls.

Fibroblast cells were cultured from skin biopsies obtained from 14 patients diagnosed with Male Idiopathic Osteoporosis and from 13 healthy age-sex matched controls, without a diagnosis of osteoporosis. Transport of the amino acid tryptophan across the cell membrane was measured by the cluster tray method. The kinetic parameters, maximal transport capacity (V_{max}) and affinity constant (K_m) were determined by using the Lineweaver-Burke plot equation.

The results of this study have shown a significantly lower mean value for V_{max} ($p = 0.0138$) and lower K_m mean value ($p = 0.0009$) of tryptophan transport in fibroblasts of MIO patients compared to the control group. A lower V_{max} implied a decreased tryptophan transport availability in MIO patients.

In conclusion, reduced cellular tryptophan availability in MIO patients might result in reduced brain serotonin synthesis and its endogenous levels in peripheral tissues, and this may contribute to low bone mass/formation. The findings of the present study could contribute to the etiology of idiopathic osteoporosis and for the development of novel approaches for diagnosis, treatment and management strategies of MIO.

1. Introduction

Osteoporosis in young and middle-aged men, in the absence of secondary causes, is sometimes called “idiopathic osteoporosis”. The etiology is heterogeneous but several studies, including our own, have shown signs of osteoblast dysfunction and bone histomorphometry variables indicating low bone formation (Chavassieux and Meunier, 2001; Pernow et al., 2009; Pernow et al., 2006). Hormonal dysfunctions including mutations or polymorphisms in the aromatase, the estradiol receptors or IGF-1 system have been suggested as possible pathophysiological pathways (Khosla et al., 1998; Orwoll, 1998; Gennari and

Bilezikian, 2013; Bilezikian, 1999).

We have previously reported that Male Idiopathic Osteoporosis (MIO) patients have decreased erythrocyte tryptophan levels, compared to healthy controls. We found a positive correlation between the bone histomorphometric parameters (wall thickness, trabecular thickness and mineral apposition rate) and the erythrocyte tryptophan levels in the MIO patients. There was no difference in plasma tryptophan levels indicating a difference in tryptophan transport over the cell membrane (Pernow et al., 2010). Tryptophan is an essential amino acid that belongs to the group of large neutral amino acids (LNAA) and it is the precursor of serotonin (5-hydroxytryptamine, 5-HT). Serotonin is

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synthesized in a two-step pathway from L-tryptophan by the rate limiting enzyme tryptophanhydroxylase (Tph). Inhibition of Tph2 results in decreased production of serotonin in the brainstem. A lower central serotonin level increases the sympathetic outflow and an increased activation of osteoblast beta adrenergic receptors results in lower bone formation (Niedzwiedzki and Filipowska, 2015). It has been demonstrated in an animal model, that chronic tryptophan deficiency results in decreased serotonin synthesis, which can lead to low bone mass and low bone formation (Sibilia et al., 2009). The brain serotonin production is dependent on the amount of tryptophan transported over the blood brain barrier (BBB). Thus, a lower tryptophan transport capacity could result in lower bone formation. The rate of serotonin synthesis depends on affinity of tryptophan to the transporter protein and the transport of tryptophan across the plasma membranes. In the human fibroblast cell model, tryptophan transport has been functionally characterized (Vumma et al., 2011). Tryptophan is mainly through LAT1 isoform of system-L through the fibroblast cell membranes at different concentrations ranges (Vumma et al., 2011). Skin derived fibroblast cells have been used as an in-vitro cell model in many studies to examine the transport of amino acids across cell membranes of patients with schizophrenia, bipolar disorder and autism (Hagenfeldt et al., 1987; Flyckt et al., 2001; Persson et al., 2009; Johansson et al., 2011). The aim of the present study was to investigate the kinetic parameters of tryptophan transport in fibroblasts obtained from men with idiopathic osteoporosis and from healthy controls.

2. Materials and methods

2.1. Study subjects

Fourteen men with idiopathic osteoporosis were recruited from the Department of Endocrinology, Karolinska University Hospital. The median age was 56 years (range 39–68). Osteoporosis was defined as 2.5 SD below the mean Bone Mineral Density (BMD) of young healthy men at the lumbar spine or hip. There was a history of osteoporotic fractures in 10 patients; 4 had vertebral fractures, one had hip fracture and 5 had other peripheral fractures. The reason for the finding of osteoporosis in the 4 patients without fractures was familial disposition. Eight patients had a family history of osteoporosis. None of the patients had secondary osteoporosis, i.e. endocrine disorders, malabsorption syndromes, alcoholism or medications known to interfere with bone metabolism. Three patients with osteoporosis had been included in the previous study on amino acids in Male Idiopathic Osteoporosis (Pernow et al., 2010). Osteoporosis was diagnosed at admittance to the hospital, in some cases several years prior to the present study. The T- and Z-score at the time of diagnosis are given in the clinical presentation of the patients as well as previous or ongoing medical treatment for osteoporosis. For patients BMD at lumbar spine and femoral neck were measured with Hologic QDR 4500 DXA (Hologic, Waltham, MA, USA) and Lunar DPX-L or Lunar Prodigy (Lunar, Madison, WI, USA). The data are listed as T-score; standard deviation (SD) from the mean of normal young adult males according to a reference population provided by the manufacturer (Lunar: USA reference population v101, Hologic: NHANES) and Dual Energy X-Ray Absorptiometry, Lunar Prodigy Advance PA + 130198.

Fibroblast cell lines derived from the skin biopsies of patients. One biopsy was lost in preparation. The presented data is from 14 patients with MIO (Table 1). The excluded patient did not differ in clinical presentation from the other MIO patients.

Fourteen healthy age-matched male volunteers were recruited from the university staff, with a median age of 56 (range 42–66) served as controls (Table 1). Interviews revealed no significant previous or present illness and they took no medication. One of age-matched controls (ID-code: FFP-O-20) showed to have osteoporosis and has been excluded from the study. The presented data is from 13 healthy age-matched male controls (Table 1).

Table 1
Characteristics and bone mineral density of male osteoporotic patients and age-matched control men.

	MIO (n = 14)	Control (n = 13)	p-Value
Age at study	56 ± 11	55 ± 8	ns
Age at diagnose	48 ± 9	na	
Body weight (kg)	78 ± 7	84 ± 17	ns
Body height (cm)	180 ± 6	180 ± 3	ns
BMI (kg/cm ²)	24 ± 2	26 ± 5	ns
Family history of osteoporosis	8 yes 5 no 1 na	None	
Fractures	10 yes 4 no	1 yes 12 no	
BMD lumbar spine			
Tscore, mean ± SD	-3,0 ± 0,7 (n = 13)	0,6 ± 1,5	p < 0,001
Median (range)	-3,3 (-4,4 to -1,9)	0,2 (-1,5 to 2,7)	
Zscore, mean ± SD	-2,7 ± 0,7 (n = 13)	0,7 ± 1,2	p < 0,001
Median (range)	-2,8 (-3,9 to -1,8)	0,4 (-1,8 to 2,4)	
BMD femoral neck			
Tscore, mean ± SD	-2,4 ± 1,1 (n = 13)	-0,6 ± 1,1	p < 0,001
Median (range)	-2,6 (-4,1 to 0,0)	-1,3 (-1,6 to 1,3)	
Zscore, mean ± SD	-1,7 ± 1,1 (n = 13)	0,04 ± 0,9	p < 0,001
Median (range)	-2,0 (-3,5 to 0,9)	0,0 (-1,5 to 1,6)	
Treatment for osteoporosis	10 yes (bisphosphonates) 4 no	No	

Notes: variables are shown as mean ± S.D. Abbreviation: (n =), number of subjects, na: not available; ns: not significant; S.D.: standard deviation; BMI: body mass index; BMD: Bone mass density. Z-score = (observed BMD - mean BMD for age- and sex-matched healthy subjects)/SD for age- and sex-matched healthy subjects. T-score: BMD in a patient compared to young normal controls. Osteoporosis is defined as 2.5 SD below the mean BMD of young healthy men at the lumbar spine or hip.

2.2. Materials

All growth media used for cell culture, antibiotics and fetal bovine serum (FBS) were obtained from Gibco Invitrogen (Sweden). Tissue culture flasks and multi-well plates were from Costar Europe Ltd., Costar NY. [3H] L-tryptophan with a specific activity of 20 Ci/mmol was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). D-Glucose was purchased from Ambresco (Ohio, USA) and phosphate buffered saline (PBS) was obtained from the National Veterinary Institute (SVA) (Uppsala, Sweden). Scintillation cocktail (Optiphase, Hisafe 3) and liquid scintillation counter (Winspectral1414) were from Perkin Elmer Life Sciences (USA). Scintillation vials were purchased from Sarstedt AB (Sweden). 96-well plates used for protein determination were purchased from Nunc (Roskilde, Denmark). The tryptophan solutions were made in PBS and the pH was maintained between 7.35 and 7.40.

2.3. Cell culture

Fibroblast cells were cultured in plastic tissue culture flasks containing minimal essential medium (MEM) supplemented with 10% FBS, L-glutamine (2 mM/l), penicillin (100 mg/ml), streptomycin (100 mg/ml) and Amino-Max™. Cells were maintained in a humidified atmosphere of 5% CO₂ in air at 37 °C. Confluent cells harvested and seeded in to 2 cm² multi-well plates and grown till confluent for approximately 5 days were used for carrying out tryptophan transport assays. Cell lines between 4th and 15th passages (number of splitting) were used in the experiments.

2.4. Tryptophan transport

Kinetic parameters (V_{\max} and K_m) for tryptophan transport were determined at concentrations ranging from 5 μM to 2 mM. Tryptophan transport was measured using the cluster tray method for rapid measurement of amino acid flux in adherent fibroblast cells (Hagenfeldt et al., 1987; Johansson et al., 2011; Vumma et al., 2008). Fibroblasts grown in multi-well plates were washed twice with PBS and incubated with PBS containing 1% D-glucose for 1 h at 37 °C, to deplete the endogenous amino acid pools. After removal of the D-glucose, the cells were incubated for 5 min at 37 °C with a constant amount of [3H] L-tryptophan and 12 different concentrations (5 μM to 2 mM) of unlabeled tryptophan in duplicates. Tryptophan transport was terminated by rapidly washing the cells twice with ice-cold PBS. The cells were then lysed in 0.2 ml of 0.5 mol/L sodium hydroxide (NaOH) for approximately 30 min. The radioactivity of the cell lysate was measured by liquid scintillation counting from a mixture of cell lysate and scintillation cocktail. The total tryptophan uptake was correlated (normalized) to the total amount of protein in each well that was determined by the Bradford method (Bradford, 1976), by measuring the absorbance of the cells lysate at 620 nm by a Multiskan FC Microplate Photometer (Thermo Scientific, Finland) and using bovine serum albumin as a standard. All assays were performed in duplicates.

2.5. Calculations

The tryptophan kinetic parameters V_{\max} and K_m were calculated from the obtained uptake values, corrected for diffusion constant (K_d), by using the Lineweaver-Burke plot equation $[1 / V_0 = (K_m / V_{\max}[S] + (1 / V_{\max}))]$, where the V_0 is the initial transport capacity, $[S]$ is the substrate concentration, V_{\max} is the maximal transport capacity (nmol/min/mg protein) and K_m , is the affinity constant (the concentration at half-saturation, $\mu\text{mol/l}$). Each experiment was performed in duplicate at the same time point for all tryptophan's transport assays and a mean value was taken for kinetic analysis.

2.6. Statistical analysis

All kinetic parameters are presented by descriptive statistics (mean with standard deviations). Assumptions about parametric methods were fulfilled for V_{\max} and K_m of tryptophan transport (determined using D'Agostino & Pearson omnibus normality test). No outliers were identified when tested by ROUT method setting Q to 1%. Significance of the difference in V_{\max} and K_m for tryptophan transport between osteoporosis patients and the control group were analyzed using the parametric Student's unpaired *t*-test. For all statistical analyses a significant level of 5% (two-tailed) was accepted. All statistical analyses were performed using Graphpad Prism 6 for mac.

Statistically significant differences have been indicated by asterisks: * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$.

2.7. Ethical consideration

The present study was approved by the Regional Ethics Committee in Karolinska institute, Sweden. A written and signed consent was obtained from the patients and the healthy controls before collecting the skin biopsies. The samples handling and the experiments were performed in accordance to Good Clinical and Laboratory Practice (GCP & GLP) guidelines and Helsinki's declaration.

3. Results

3.1. Bone mineral density in MIO patients and age-matched control

In the patient group, at diagnosis, the mean T-score at the lumbar spine was -3.0 SD with a range from -1.9 to -4.4 SD. Ten out of

Table 2

Kinetic parameters (V_{\max} , K_m) of tryptophan transport in fibroblasts from MIO patients and matched controls.

Kinetic parameter	MIO patients (n = 14), mean (SD)	Matched controls (n = 13), mean (SD)	p-Value (p < 0.05)
V_{\max}	0.97 (0.24)	1.69 (0.89)	0.0138*
K_m	12.97 (4.98)	20.28 (5.12)	0.0009***

The results are presented as mean (SD). (n =), number of subjects. MIO, indicates Male Idiopathic Osteoporosis. V_{\max} , indicates maximal transport capacity of tryptophan (nmol/min/mg protein) and K_m , indicates affinity of binding sites for tryptophan ($\mu\text{mol/l}$). Statistically significant differences: * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$.

fourteen had a history of at least one osteoporotic fracture. The mean T-score at the femoral neck was -2.4 with a range from -4.1 to 0 SD. The included matched control males had normal Bone Mineral Density. A summary of the results of Bone Mineral Density in patients and controls are presented in Table 1.

3.2. Tryptophan kinetics in MIO patients and matched controls

Maximal transport capacity (V_{\max}) and mean affinity of binding site (K_m) of tryptophan transport in MIO patients and in controls are presented in Table 2. The resulted mean value of V_{\max} (0.97 (0.24) nmol/5 min mg. protein) of the MIO patients was significantly lower ($p < 0.05$) than the mean value of V_{\max} (1.69 (0.89) nmol/5 min mg. protein) of the matched controls. Similarly, the mean K_m value of tryptophan transport for MIO patients (12.97 (4.98) $\mu\text{mol/l}$) was significantly lower ($p < 0.005$) than the mean K_m value in the matched controls samples (20.28 (5.12) $\mu\text{mol/l}$).

4. Discussion

In this study we found differences in amino acid transport which could be a possible pathophysiological mechanism in the regulation of bone mass. In fibroblasts from men with idiopathic osteoporosis we could demonstrate a lower tryptophan transport compared to healthy controls. This supports our hypothesis from previous work where we found decreased levels of erythrocyte tryptophan levels in men with MIO, in contrast to plasma levels which were similar to controls. This indicated a defect in the transport of tryptophan across the cell membranes (Pernow et al., 2010). The principal finding of this study was that tryptophan transport across the fibroblast cell membranes in MIO patients had a lower K_m and V_{\max} mean values compared to the control group. The significantly decreased K_m values in MIO patients indicated a higher affinity of the tryptophan transporter protein-binding site compared to the control group. Hence, the MIO patients will need a lower concentration of extracellular tryptophan to reach maximal transport capacity. A low V_{\max} implies that the transport systems have lower capacity for amino acid uptake. Hence, the MIO patients group had a decreased transport of tryptophan.

One pathway by which tryptophan may influence bone formation was demonstrated in the studies of leptin and serotonin by Yadav and coworkers (Yadav et al., 2010; Karsenty and Gershon, 2011; Gershon and Tack, 2007). The serotonin synthesized in the brain is dependent on the amount of tryptophan transported over the blood brain barrier and the activity of the tryptophan hydroxylase (Tph2). Serotonin decreases the sympathetic output and the adrenergic activation of the osteoblasts, which results in increased bone formation. The role of serotonin in the regulation of bone is complex. Increased production of serotonin in the brainstem is associated with increased bone formation whereas increased levels of peripheral serotonin stimulate bone resorption (Niedzwiedzki and Filipowska, 2015; Karsenty and Gershon, 2011).

The tryptophan transport in the fibroblast has been functionally characterized and is mainly transported through LAT1 isoform of

system-L across the fibroblasts cell membrane (Vumma et al., 2011). However, system-L also mediate uptake for at least 10 other amino acids (Palacin et al., 1998; Prasad et al., 1999). These amino acids would together present at mill molar concentration in plasma, and could saturate the binding sites of transporters proteins of the system L and act as competitive inhibitors of tryptophan uptake (Seymour et al., 2006). Our previous study has demonstrated elevated plasma levels of non-essential amino acids (NEAA) in MIO patients compared to healthy controls (Pernow et al., 2010), and that might result in lower tryptophan levels compared to the other amino acids, i.e. alanine tryptophan and glycine transported by the same isoform (Johansson et al., 2011), but they are transported in different fractions by system-L and could compete at the level of system-L. Under these circumstances, a high affinity transport system for tryptophan would be a compensatory mechanism to the competitive inhibitory effects of the presence of other amino acids.

In the present study, the finding of increased affinity of binding site of the transporter proteins for tryptophan in MIO could be a compensatory mechanism to the decreased Vmax. The increased affinity can largely be explained by alteration of LAT1 isoform properties (such as different light-chain subunits) of existing system-L that confer enhanced affinity for tryptophan (Verrey, 2003). Alternatively, it is possible that a novel system unrelated to existing transporters is involved in the new activity.

Since tryptophan is the precursor for the synthesis of serotonin, decreased tryptophan transport across the cell membranes could result in reduction of serotonin synthesis at cellular levels. The decreased transport of tryptophan in fibroblasts have been previously reported in many in vitro studies for disorders such as ADHD and autism, which can lead to decreased levels of serotonin in the CNS (Johansson et al., 2011). Human osteoblast cells and osteosarcoma cell lines express LAT-1 and LAT-2 (Kim et al., 2006a) and increased LAT1 expression in early bone formation has been reported (Kim et al., 2006b). Decreased tryptophan transport may have a direct effect on bone cells. Inhibition of Trh1 in mice has been shown to counteract ovariectomy induced osteoporosis (Yadav et al., 2010) and the stipulated mechanism by decreasing gut-derived serotonin. In line with the results from the present study, an additional mechanism could be hypothesized. By inhibiting Trp1 the available tryptophan increases at the cell level and for membrane transportation, and could thus increase brain serotonin.

The present study has limitations. This study included a small number of cell lines (i.e. fourteen) of clearly selected osteoporotic patients. They were diagnosed with osteoporosis at a fairly young age and many had a family history of osteoporotic fractures. Performed power calculations shown that the sample size used in this study assures an adequate power to detect the statistical significance.

In conclusion, we demonstrate that male patients with idiopathic osteoporosis have decreased fibroblast tryptophan transport which is in accordance with our previous finding of low erythrocyte tryptophan levels. The reduced cellular tryptophan availability in MIO patients might result in reduced brain serotonin synthesis and its endogenous levels in peripheral tissues, this may contribute to low bone mass. Nevertheless, further studies are needed to evaluate the possible pathophysiological mechanisms.

However, tryptophan transport and its physiological relevance in MIO have not been investigated before and need further characterization such as competition, molecular and in vivo studies. The relative importance of mutations and/or polymorphism in the L-system in the regulation of bone mass needs to be confirmed in larger cohorts of osteoporotic patients.

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Conflict of interest disclosure

The authors do not have anything to disclose.

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